

*AMENDMENTS TO THE SPECIFICATION*

*Please replace paragraph [0030] with the following replacement paragraph.*

[0030] B) Vessels are used for the wash process such that damage to the cells going in and out of the washes is minimized without compromising either the physical contact of pine cells with the wash media (which is responsible for the eradication of bacterial cells), or the air-media interface area and thus aerobicity of the cultures during the wash period. An improvement over vessels commonly used for washing procedures, such as flasks, wherein the cells can be crushed or lost in the cumbersome process of passing into such wash containers, consists of using wide-mouthed jars, most preferably with aerated lids that maintain axenic conditions while providing aeration to the pine cells. An example of such vessels comprises “baby food” jars with MAGENTA® aerated lids (available from SIGMA), most preferably in a size such that they can be fixed with standard clamps similarly to flasks for agitation on automatic shakers, providing further aeration of the pine cells and maximizing physical contact of the pine cells with the wash media.

*Please replace paragraph [0034] with the following replacement paragraph.*

[0034] In this improvement, the target pine cells are cultured following transformation on polyester or fluoropolymer support membranes placed over gelled support media containing a selection agent. Experiments were conducted to determine whether selection agents (such as kanamycin, GENETICIN® antibiotic, herbicides, and the like) would be able to pass from the underlying medium through non-cellulosic support membranes to tissue in contact with the membrane. Our results indicate that the selection agents kanamycin, GENETICIN® antibiotic and various herbicides of interest were able to pass through polyester support membranes, selecting tissue which has been transformed with a kanamycin, GENETICIN® antibiotic or herbicide

resistance gene by killing tissue which has not been transformed with this gene. Indeed, the incidence of "escapes" (i.e., untransformed cells which fail to be killed by the selection agent) was found to be lower when polyester support membranes were employed in the selection method than when the cells are cultured directly on the surface of media containing the selection agent, or when filter paper or nylon supports were used.

*Please replace paragraph [0058] with the following replacement paragraph.*

[0058] After megagametophyte explants were placed in culture, the perimeter of the dish was sealed with two wraps of NESCOFILM® sealing film (commercially available from Karlan Company). The dishes were incubated in the dark at a constant temperature of  $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . After about 7 to 21 days, embryogenic tissue extruded from the micropyle of the megagametophyte explants. At six weeks following the placement of the explant on initiation media, tissue masses that had extruded and were proliferating from individual explants were isolated to individual petri plates on maintenance medium DCR<sub>2</sub> or WV5<sub>2</sub> and assigned line numbers. After one to three months of culture on maintenance medium, the tissue cultures were cryopreserved.

*Please replace paragraph [0060] with the following replacement paragraph.*

[0060] Frozen cultures were retrieved when desired by removing individual vials from the cryobiological storage vessel and placed in  $42^{\circ} \pm 2^{\circ}\text{C}$  water to rapidly thaw the frozen cell suspensions. The thawed cell suspensions were aseptically poured from the cryovial onto a sterile 35  $\mu\text{m}$  pore size polyester membrane support placed over sterile filter paper (~~Whatman~~ WHATMAN® filter paper no. 2, Whatman International Ltd.) for a few minutes to allow the DMSO cryoprotectant solution to diffuse away from the embryogenic tissue into the paper. The embryogenic tissue on the polyester support membrane was then transferred to DCR<sub>2</sub> maintenance

medium and incubated at 23°C in the dark for 24 hours to allow additional DMSO to diffuse away from the tissue into the medium. The polyester support bearing the embryogenic tissue was then removed from the medium and transferred to fresh DCR<sub>2</sub> maintenance medium, and thereafter, every 14-21 days to a fresh plate until the amount of cells per plate reached about 1 g. The culture environment during post-cryopreservation recovery and growth was 23°C  $\pm$  2°C in the dark. Those skilled in the art will recognize that many different cryopreservation and recovery procedures would be suitable for use with this method and the detail in this example may not be construed to limit the application of the method.

*Please replace paragraph [0063] with the following replacement paragraph.*

[0063] Following co-cultivation, the eradication of *Agrobacterium* from the cultures was carried out as follows. The cells were re-suspended into fresh DCR<sub>4</sub> liquid wash medium (Table 2), which in some treatments contained eradicates such as 200-400 mg/L TIMENTIN® antibiotic, 250-500mg/L carbenicillin, or 250-500mg/L cefotaxime. Those skilled in the art of plant transformation will recognize that a variety of different eradicates may be used against *Agrobacterium*, and any of those are suitable for the present method. The DCR<sub>4</sub> liquid wash medium was contained in sterile containers comprising either conventional Erlenmeyer flasks, Nephelo sidearm flasks as described above, screw-top test tubes, MAGENTA® boxes with conventional lids or MAGENTA® aerated lids, "baby food" jars with conventional lids or MAGENTA® aerated lids, conventional beakers, or multi-well plates. Resuspension was initiated by grasping the membrane support bearing the infected cells, using forceps, and rolling or folding it so that it could be taken up and placed into the liquid in the wash container. The liquid was then agitated to get the cells into suspension, and the membrane support was scraped with sterile forceps if cells appeared to be adhering to it. Once the cells were in suspension, the membrane was removed with sterile forceps.

*Please replace paragraph [0074] with the following replacement paragraph.*

[0074] Embryogenic cell lines of *P. taeda*, as well as cell lines of hybrids between *P. taeda* and *P. rigida*, used in this example, were generated by the methods described in the above example. Two hundred proliferating culture lines were selected for use in this study and randomly assigned to one of two treatments using a very small amount of tissue, to simulate the situation following identification of a transformation event on selection:

A: Approximately 0.1 g of tissue was placed directly onto the surface of the gelled maintenance medium.

B: Approximately 0.1 g of tissue was placed onto a polyester support membrane (SEFAR PeCap® fabric support Catalog No. 7-35/11) cut to 55 mm square, laid on the surface of the gelled maintenance medium.

*Please replace paragraph [0088] with the following replacement paragraph.*

[0088] Loblolly and hybrid pine cell lines which had been grown and maintained as described in Examples 1-2 above were used in this example. Support membranes bearing pine tissue were placed on gelled DCR<sub>2</sub> maintenance media with various antibiotics (cefotaxime or TIMENTIN® antibiotic) incorporated into the gelled DCR<sub>2</sub> maintenance media, or into liquid DCR<sub>4</sub> pipetted in a thin film over gelled DCR<sub>2</sub> maintenance media lacking antibiotics, or into liquid DCR<sub>4</sub> which was saturated into a filter paper laid on gelled DCR<sub>2</sub> maintenance media lacking antibiotics. Support membranes bearing control cells were placed either on gelled DCR<sub>2</sub> maintenance media, over liquid DCR<sub>4</sub> pipetted in a thin film over gelled DCR<sub>2</sub> maintenance media lacking antibiotics, or over a filter paper saturated with liquid DCR<sub>4</sub> and laid on gelled DCR<sub>2</sub> maintenance media lacking antibiotics. The eradication treatments and controls were continued for a period of approximately

12 weeks, with transfer of the polyester support membranes, bearing the pine embryogenic cells, every 14-21 days.

*Please replace paragraph [0091] with the following replacement paragraph.*

[0091] In a further test, this time using pine cells treated with *Agrobacterium* as described in Example 1 above, that therefore did require eradication, were plated on the treatments described above. In this case, eradicans used were TIMENTIN® antibiotic at higher concentrations (either 400, 500 or 800 mg/L) and AUGMENTIN® antibiotic at 500 mg/L. Eradicants presented to the cells in liquid DCR<sub>4</sub> pipetted in a thin film over gelled DCR<sub>2</sub> maintenance media, or saturated into a filter paper laid on gelled DCR<sub>2</sub> maintenance media were as or more successful in suppressing the growth of *Agrobacterium* than eradicans incorporated in the gelled DCR<sub>2</sub> media, with the overall use of only 7.5% of the amount of eradican per plate in which it was applied (1.5 ml liquid vs. 20 ml gelled medium).

*Please replace paragraph [0095] with the following replacement paragraph.*

[0095] *Agrobacterium* contamination has been reported as recurring sometimes after long periods of time. With many species, eradicans are incorporated in all culture media used after the initial infection, including selection media, proliferation media, media to induce the formation of organs or the development of somatic embryos, media to elongate or mature organs or embryos that are formed, and regeneration media. For pine embryogenic cells, incorporation of eradicans into the embryo development and maturation media has been difficult due to the high temperature of polymerization of the media resulting from the incorporation of a high level of polyethylene glycol. Therefore, loblolly and hybrid pine cell lines grown and maintained as described in Examples 1-2 above were placed on polyester support membranes over gelled MSG<sub>1</sub> embryo development and

maturation media as described in Example 2 above, except that some of the development and maturation media were overlaid with various eradication treatments under the polyester support membranes. The treatments consisted of either no liquid phase, or a liquid phase identical to the gelled phase (except that gelling agent and activated charcoal were omitted) and incorporating either cefotaxime or TIMENTIN® antibiotic as an eradicator. Three replicate plates were generated from each of six embryogenic cell lines (two from each of two *P. taeda* families and one hybrid pine family) for each of the treatments, and assessed for the ability of the cultures to develop high quality harvestable stage 3 embryos. Dishes were incubated in a dark growth chamber at  $23 \pm 2^\circ\text{C}$ . The membrane supports were transferred to new petri dishes containing fresh medium every 3 weeks. At week 9, stage 3 embryos were counted and those deemed suitable for germination were harvested. The results are shown in Table 11 below.

*Please replace paragraph [0097] with the following replacement paragraph.*

[0097] Embryos developed during biphasic application of eradicators as described in the paragraph above were subjected to a germination test to determine whether the biphasic application of eradicators under polyester support membranes over gelled MSG<sub>1</sub> embryo development and maturation media had affected their germinability. The treatments during embryo development consisted of either no liquid phase, or a liquid phase identical to the gelled phase (except that gelling agent and activated charcoal were omitted), or the same liquid phase incorporating either 200 or 400 mg/L TIMENTIN® antibiotic as an eradicator. Germination was carried out as described in Example 2, and the results are shown in Table 12 below.

*Please replace paragraph [0098] with the following replacement paragraph.*

[0098] As shown in Table 12, and in similar results using AUGMENTIN® antibiotic or higher concentrations of TIMENTIN® antibiotic (500 and 800 mg/L), the application of a liquid phase between the gelled medium and the cells borne on a polyester support membrane, whether or not it contains eradicator, does not result in a significant detriment to embryo germination. Thus, if required due to long-term resurgence of bacterial growth in a culture, the method described in this example allows the application of antibiotics even in the presence of embryo development and maturation media in which they could not otherwise be effectively incorporated.

*Please replace paragraph [0099] with the following replacement paragraph.*

[0099] Loblolly and hybrid pine cell lines were used which had been grown and maintained as described in Examples 1-2 above. In order to test selection improvements that would be carried out alone or in combination with eradication procedures following *Agrobacterium* transformation, without confounding any growth effect related to the *Agrobacterium* gene transfer process and unrelated to the selection and eradication methods *per se*, transformed lines resistant to GENETICIN® antibiotic were generated by the bombardment method described in U.S. patent application Serial No. 09/318,136 filed on 25 May 1999 and New Zealand Patent No. 336149, each incorporated herein by reference.

*Please replace paragraph [0100] with the following replacement paragraph.*

[0100] Specifically, to prepare for gene transfer, a sterile fabric support (here NITEX® fabric support, commercially available from Sefar Inc.) was placed in a sterile Buchner funnel and one to five milliliters of embryogenic suspension was pipetted onto the fabric support such that the

embryogenic tissue was evenly distributed over the surface. The liquid medium was suctioned from the tissues using a mild vacuum. The fabric support with embryogenic tissue was removed from the Buchner funnel and placed on a GELRITE® gelling agent solidified DCR<sub>3</sub> preparation medium (Table 2) in 100 X 25 mm plastic petri dishes. Dishes were incubated in a dark growth chamber at 23°C ± 2°C for about 24-48 hours.

*Please replace paragraph [0102] with the following replacement paragraph.*

[0102] The petri dishes with the fabric support and embryonic tissues were then placed into the interior of the PDS 1000/He BIOLISTICS® device and vacuum applied to a level of 28 inches Hg. The gold particles carrying the DNA were accelerated toward the embryogenic tissue following a helium build-up and bursting regulated by a 1550 psi rupture disk. In the PDS-1000/He BIOLISTICS® device the gap between the rupture disk and the macrocarrier (gap distance) was five mm and the macrocarrier travel distance was 13 mm. Following DNA transfer the petri dishes containing the fabric support and tissues were incubated in a dark growth chamber at 23°C ± 2°C for about 24 hours. The tissues and fabric support were transferred to semi-solid maintenance medium, DCR<sub>1</sub> (Table 2) to recover from carrier particle bombardment and incubated in a dark growth chamber at 23°C ± 2°C for a period of 0-7 days (the duration depended on observation of when cell division had resumed in the pine cells). The tissues and fabric support were transferred to a selection medium, semi-solid maintenance medium DCR<sub>1</sub> containing a level of selection agent inhibitory to the growth of non-transformed cells. In this and subsequent examples the selection agent used was GENETICIN® antibiotic at 15-30 mg/L. The plates were incubated in a dark growth chamber at 23°C ± 2°C for about six to twelve weeks with the fabric supports containing the tissues being transferred to the same fresh culture medium every 2-3 weeks.

*Please replace paragraph [0106] with the following replacement paragraph.*

[0106] The 1:9 mixtures were immediately plated on polyester, nylon, or filter paper supports or placed directly on the surface of gelled medium as described in Example 2, with five replicate plates for each treatment and cell line combination. The medium used was DCR<sub>2</sub> containing 15 mg/L GENETICIN® antibiotic, hereinafter referred to as DCR<sub>5</sub> selection medium, which should kill the non-transformed tissue while allowing growth of the transformed tissue. To verify the ability of the DCR<sub>5</sub> selection medium to kill the non-transformed tissue, non-transformed cells were plated alone on the DCR<sub>5</sub> selection medium using 5 replicate plates per line per treatment in the same support treatments of nylon, polyester, filter paper, and directly on the gelled medium. To verify the ability of the DCR<sub>5</sub> selection medium to allow growth of the transformed tissue, transformed cells were plated alone on the DCR<sub>5</sub> selection medium using 5 replicate plates per line per treatment in the same support treatments of nylon, polyester, filter paper, and directly on the gelled medium.

*Please replace paragraph [0112] with the following replacement paragraph.*

[0112] Lines transformed with *Agrobacterium* and washed as described in Example 1 above were plated onto gelled DCR<sub>5</sub> selection media as described in Example 4 above, and freed of any remaining *Agrobacterium* contamination using the biphasic eradicator treatments described in Example 3 above, specifically using the antibiotic combination TIMENTIN® antibiotic at 400-800 mg/L in a DCR<sub>4</sub> liquid medium poured over the gelled DCR<sub>5</sub> selection media. Lines were transferred onto fresh selection media at intervals of 2-3 weeks. When each transformed subline had grown to approximately 2 g, the presence of the transgenes was verified using polymerase chain reaction (PCR) amplification of specific segments of the transgene DNA sequence carried out using techniques well known to those skilled in the art of molecular biology. This demonstrated that the

improved selection methods described in Example 4 above were sufficient to select transformed cells, without the number of escapes seen when the unimproved selection methods were used.

*Please replace paragraph [0118] with the following replacement paragraph.*

[0118] To test the growth of *Pinus radiata* cells over support membranes as described in Example 2 above or biphasic media as described in Example 3 above, cells of lines derived from five different *P. radiata* families were plated, as described in Example 2 above, on the following treatments:

- A. Cells were placed directly onto maintenance medium with no support filter or membrane.
- B. Cells were placed on a nylon support membrane (NITEX® fabric support with pre-wetting pore size 35, commercially available from SEFAR), and this membrane bearing the embryogenic cells was then placed on maintenance medium.
- C. Cells were placed on a polyester support membrane (PECAP® fabric support with pre-wetting pore size 35, commercially available from SEFAR), and this membrane bearing the embryogenic cells was then placed on maintenance medium.
- D. Cells were placed on a filter paper (WHATMAN® filter paper No. 3), which had been wetted with a few drops of liquid maintenance medium (the same as the gelled maintenance medium but lacking gelling agent), and this filter paper bearing the embryogenic cells was then placed on maintenance medium.
- E. Cells were placed on a polyester support membrane (PECAP® fabric support with pre-wetting pore size 35, commercially available from SEFAR), and this membrane bearing the embryogenic cells was then placed over gelled maintenance medium on which had been placed a filter paper (WHATMAN® filter paper No. 3) that had been

wetted with a few drops of liquid maintenance medium (the same as the gelled maintenance medium but lacking gelling agent).

- F. Cells were placed on a polyester support membrane (PECAP® fabric support with pre-wetting pore size 35, commercially available from SEFAR), and this membrane bearing the embryogenic cells was then placed over gelled maintenance medium on which had been placed a filter paper (WHATMAN® filter paper No. 3) that had been wetted with a full milliliter of liquid maintenance medium (the same as the gelled maintenance medium but lacking gelling agent).

The cells on their supports were weighed (cells in treatment A were weighed on a polyester support membrane and then gently scraped off the membrane using sterile forceps, or a sterile plastic one-use cell harvester, onto the gelled media) and were then maintained on the same supports, with weighing and transfer to fresh sterile gelled medium biweekly for four transfer cycles.

*Please replace paragraph [0122] with the following replacement paragraph.*

[0122] Following this co-cultivation period, cells were washed according to the method described in Example 1, using polyester support membranes, wide-mouthed “baby food” jars with aerated lids, and wash medium identical to the maintenance medium except that gelling agents were omitted and 400 mg/l TIMENTIN® antibiotic was added. The cells were washed three times, with the first washes lasting 1-2 hours and the third wash being an overnight agitation in wash medium at approximately 100 rpm.

*Please replace paragraph [0123] with the following replacement paragraph.*

[0123] The tissues and fabric support were then transferred to semi-solid maintenance medium for one week in order to observe whether *Agrobacterium* contamination resurged, followed

by transfer to selection medium. Selection media consist of nutrient media modified such that they allow preferential growth of transformed cells. For the purposes of this example, maintenance medium identical to the aforementioned medium, except for the addition of 400 mg/l TIMENTIN® antibiotic and 15 mg/l GENETICIN® antibiotic selection agent, which was shown to be inhibitory to the growth of non-transformed *P. radiata* cells, is referred to as *P. radiata* selection medium. The plates were incubated in a dark growth chamber at  $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for about six weeks with the fabric supports containing the tissues being transferred to the same fresh culture medium every two weeks.

*Please replace paragraph [0127] with the following replacement paragraph.*

[0127] Loblolly pine cell lines were used which had been grown and maintained as described in Example 1 above, and prepared for biolistic transformation as described in Example 4 above. Following bombardment the support membranes bearing the bombarded embryogenic cells were transferred to DCR<sub>2</sub> maintenance media for one week. Following this the support membranes bearing the bombarded embryogenic cells were divided among plates containing gelled DCR selection medium either with or without the addition of 10 mg/l abscisic acid (ABA), and cultured for three weeks. Cells selected on either type of selection medium during this first selection period on selection medium containing ABA were then divided among plates with and without ABA and cultured for a further three weeks. The same occurred in the next transfer, resulting in groups of cells that had been selected entirely in the absence of ABA, entirely in the presence of ABA, or in the presence of ABA for one of the three three-week selection periods. After the nine weeks of selection, the plates were examined for sublines growing in the presence of the GENETICIN® antibiotic selection agent, and cells from these sublines were observed for staining indicating the presence of the *uidA* transgene. The cells were also checked for the presence of sequences by PCR amplification using primers specific for both the *uidA* and *nptII* transgenes, and subsequently these

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results were further confirmed by Southern blotting, all techniques well known to those skilled in the art of plant transformation.